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PRINCIPAL INVESTIGATOR: Ya-Ling Wu
Geoffrey L. Greene, Ph.D.

CONTRACTING ORGANIZATION: The University of Chicago
Chicago, IL 60637

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6. AUTHOR(S)
Ya-Ling Wu
Geoffrey L. Greene, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of Chicago
Chicago, IL 60637

E-Mail: yalingw@midway.uchicago.edu

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13. ABSTRACT (Maximum 200 Words)

Tamoxifen is effective for the prevention and treatment of estrogen-dependent breast cancers, but is associated with an increased incidence of endometrial tumors. We completed the first aim of this proposal to solve the crystal structure of the estrogen receptor alpha ligand-binding domain (ERαLBD) bound to the structurally similar compound GW5638, which has therapeutic potential and does not stimulate the uterus. Like tamoxifen, GW5638 relocates the carboxy-terminal helix (H12) to the known coactivator-docking site in the ERα LBD. However, GW5638 repositions residues in H12 through specific contacts with the N-terminus of this helix. In contrast to tamoxifen, the resulting increase in exposed hydrophobic surface of ERα LBD correlates with a significant degradation of ERα in MCF-7 cells. Thus, the GW5638-ERa LBD structure reveals a unique mode of SERM-mediated ER antagonism, in which the stability of ERα is decreased through an altered position of H12. This dual mechanism of antagonism may explain why GW5638 can inhibit tamoxifen-resistant breast tumors. In addition, difficulties encountered with experiments under aim 2 and 3 were addressed along with alternative approaches proposed in this report.

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ANNUAL SUMMARY TRAINING REPORT FOR SET 01, 2003– AUG 31, 2004

INTRODUCTION

Breast cancers affect one in eight women in the United States. Many of these cancers respond to hormonal therapy and the presence of estrogen receptor (ERα) is associated with a more favorable response and short-term prognosis (Hunt, 1994). Tamoxifen or 4-hydroxytamoxifen (OHT) is part of a growing family of molecules called selective estrogen receptor modulators (SERMs) that can behave as agonists or antagonists in different tissue and environmental contexts. It has been widely used for breast cancer treatment and shows considerable potential as a preventive agent. Unfortunately, advanced breast cancers that initially respond well to tamoxifen eventually become refractory to this compound. Its uterotrophic activity also restricts its utility in a prevention setting (McDonnell et al., 2002). GW5638 is a novel SERM that differs from tamoxifen in that the dimethylaminoethyoxy group is replaced by an acrylate side chain (Willson et al., 1994). This compound exhibits beneficial estrogenic properties but unlike tamoxifen, it is a more potent antagonist in breast cancer cells and has no uterotrophic behavior. Because tamoxifen-resistant breast cancers are not cross-resistant to GW5638, this SERM has significant potential as a therapeutic agent. GW5638 and its 4-hydroxy metabolite (GW7604) can induce a unique conformational change in ERα that is recognized by synthetic peptides selected by phage display. These peptides recognize GW5638/GW7604-ERα complexes but not tamoxifen-ERα or other ligand-bound ER complexes, indicating that conformational changes elicited by GW5638 and tamoxifen are different (Connor et al., 2001). To better understand the pharmacology of GW5638, we proposed (1) to determine the structure of human ERα/β-LBD in complex with GW5638 (2) to determine the structure of ERα/β-LBD/GW5638 associated with GW 7β-16 and/or other CoRNR box peptides. In addition, we also propose to determine the structure of C through E domains of human ERα/β and to characterize the interactions among these functional domains, which may have important pharmacological significance in biology and drug design.

BODY

Aim 1: To determine the structure of human ERα/β-LBD in complex with GW5638 and to compare the conformational changes in ERα/β-LBD elicited by tamoxifen and GW5638.

I have completed this specific aim, which includes (1) generating and purifying GW-ERα LBD for biochemical analysis and crystallization; (2) obtaining single, diffraction-quality crystals for x-ray analysis; (3) determining the x-ray structure of GW-ERα LBD and depositing the structure to the protein data bank.

Details of each specific aim are given as follows:

(1) Generating and purifying GW-ERα LBD for biochemical analysis and crystallization

The human ERα LBD (residues 297–554) was expressed in BL21(DE3)pLysS as described previously (Shiau et al., 1998). The amount of ERα LBD in each bacterial extract is determined by saturation analysis with ^{3}H-estradiol using a controlled pore glass bead (CPG) assay. Protein purification is carried out on an estradiol-Sepharose affinity column (E-Seph) (Greene et al., 1980), followed by FPLC ion exchange purification (Resource Q, Pharmacia). While still bound to the E-Seph beads, ERα LBD is caboxymethylated with 5 mM iodoacetic acid (Hegy et al., 1996). GW5638-containing buffer is used for eluting ERα LBD from the affinity column. Protein samples are than analyzed by native gel electrophoresis. FPLC fractions containing the purest sample was collected and concentrated to 5mg/mL-10mg/mL for crystallization.

(2) Obtaining single, diffraction-quality crystals for x-ray analysis

The crystals of GW5638-hERα LBD were grown at 4°C by hanging drop vapor diffusion. Samples (2 μl) of 5 mg/ml protein were mixed with 2 μl of the reservoir buffer consisting of 1.5%-2% ethylene imine polymer, 100 mM tri-sodium citrate (pH 5.6-pH 5.7), 0.5 M of sodium chloride and 9% of Yttrium chloride hexahydrate. Crystals that grew up to the size of >0.1 mm were harvested and transferred to a cryoprotectant solution containing 2% ethylene imine polymer, 100 mM tri-sodium citrate (pH 5.6), 1M of sodium chloride and 25%-40% glycerol and stored in liquid nitrogen. Data were collected at BioCARS 14BMC beamline (λ = 0.9 Å) station, Advanced Photon Source, Argonne National Laboratory. Diffraction data were recorded on an ADSC Quantum-4 detector. The images of data sets were processed with Denzo and Scalepack programs in the HKL 1.96 package.

(3) Determining the x-ray structure of GW-ERα LBD and depositing the structure to the protein data bank

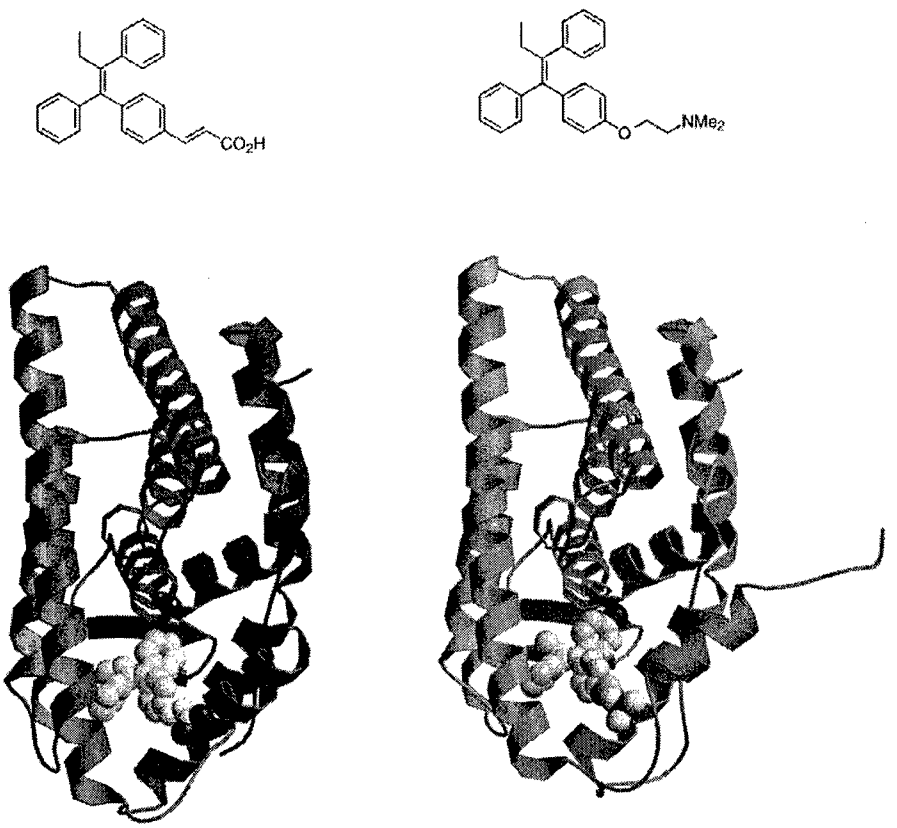
Our initial efforts to determine the structure utilized a low-resolution (3.3 Å) data set. The three ligand-binding domains in the asymmetric unit were located by molecular replacement with EPMR version 2.5 (Kissinger et al., 1999) using a modified raloxifene-hERα LBD (1ERR.pdb) as the search probe. The crystals of the GW5638-hERα LBD lie in the space group of $P6_122$. The unit cell parameters are a = b = 136.031 Å and c = 357.626 Å. The R factor and correlation coefficient (CC) after rigid body refinement are 43.1% and 0.65, respectively. The model was refined initially with CNS 1.0 (Brunger et al., 1998) and later with Refmac5.1.24 (Murshudov et al., 1997). TLS restrain (Schomaker and Trueblood, 1968) in Refmac was applied to correct for anisotropic displacements of each monomer in the asymmetric unit. Model building of the GW5638-hERα LBD structure that was not included in the original search probe from molecular replacement was done by Xtalview (McRee, 1999). The R_{free} set contains a random sample of 10.2% of all data. The structure of GW5638-hERα LBD using data up to 2.7 Å is refined to a crystallographic R factor of 20.8% and an R_{free} factor of 23.6%. The coordinates of this structure have been deposited in the Protein Data Bank (PDB ID: 1R5K). The statistics of the structure and data sets are summarized in Table 1.

The crystal structure reveals a unique LBD conformation in which AF2 H12 is repositioned by direct contacts between the carboxyl side chain of GW5638 and the N terminus of H12 (Figure 1 - Figure 3). I have also performed the surface hydrophobicity as well as the ERα stability study to confirm the structural information. In contrast to tamoxifen, the resulting increase in exposed hydrophobic surface of ERα LBD correlates with a significant degradation of ERα in MCF-7 cells, which may account for the ability of GW5638 to inhibit tamoxifen resistant MCF-7 breast tumor explants.

Table 1. Statistics for GW5638-hERα LBD structure determination

Data Collection Details	
Wavelength	λ=0.9
Space Group	P6(1)22
Unit Cell Dimension	a = b = 136.031 Å; c = 357.626 Å
Processing Statistics	
Resolution Range	100-2.70 Å
Observations	55075
Unique reflections[a]	53588 (5227)
Completeness[a]	97.3% (96.8%)
I/σ (last shell)	26.2 (2.1)
R_{merge}[b]	7.2%
Refinement Statistics	
Resolution Range (Å)	27.95-2.70 (2.77-2.70)
Reflections Used (R_{free} set)	45583 (5160)
Total non-hydrogen atoms	5759
R_{cryst}(%)[a, c]	20.8 (34.8)
R_{free}(%)[a, c]	23.6 (35.5)
R.m.s.d. deviation	
Bonds (Å)	0.014
Angles (°)	1.34
Average B-factor ($Å^2$)	45.9

Figure 1. Overall structure of the GW-hERα LBD compared to the OHT-hERα LBD



GW5638 **OHT**

Figure 2. Superimposed structures of GW5638-hERα LBD (blue cylinder) and OHT-hERα LBD (yellow cylinder)

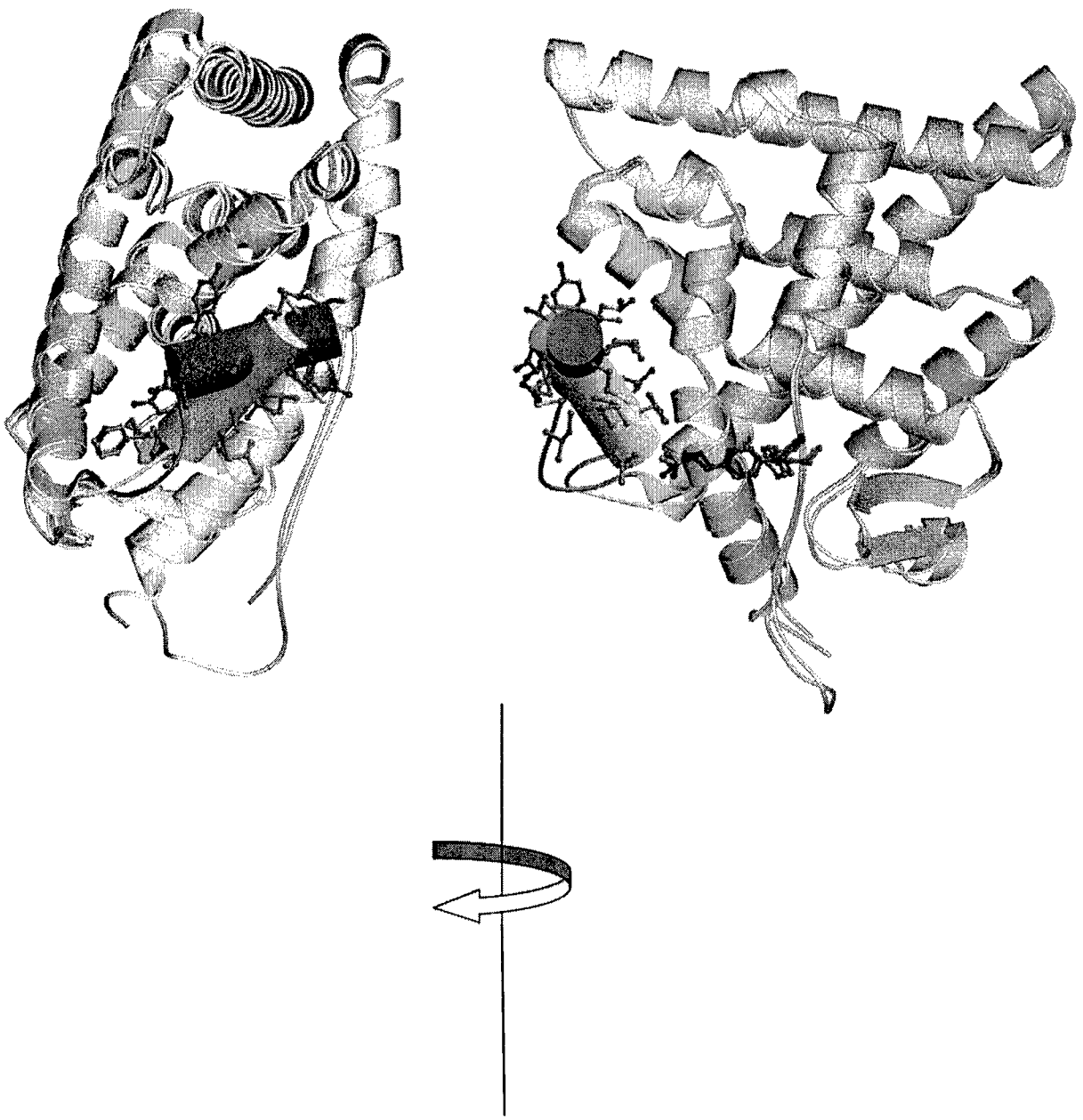
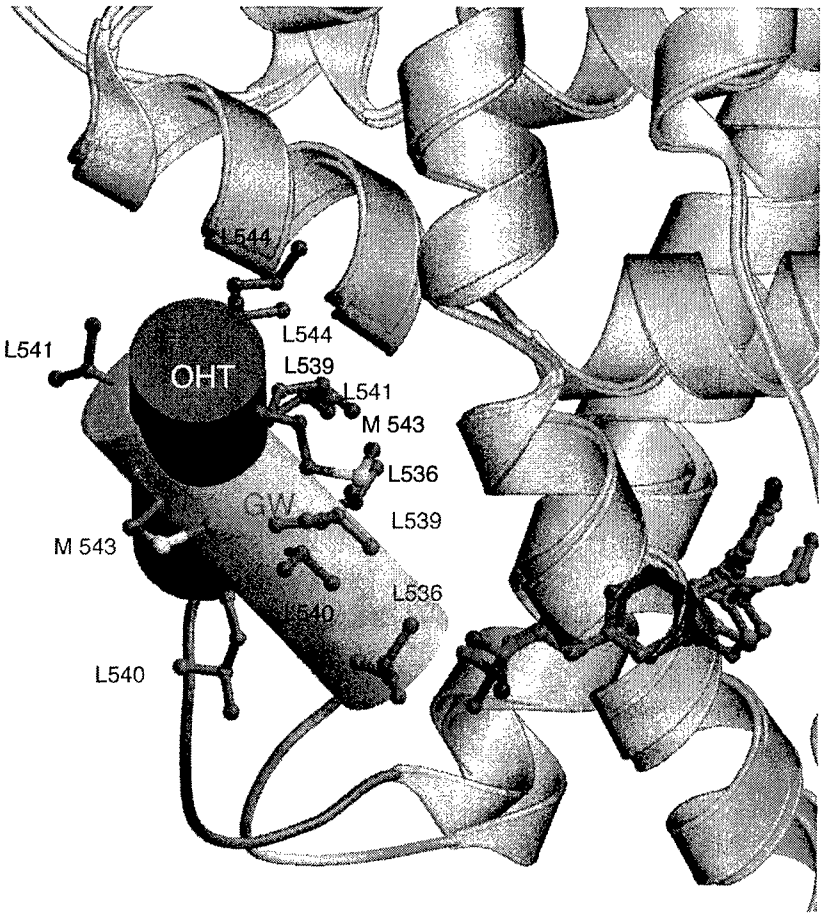


Figure 3. A closer look of the superimposed GW and OHT-hERα LBD structures. Only hydrophobic residues in H12 are shown. Side chains and ligands are colored by atom type (carbon (GW) = green, carbon (OHT) = blue, nitrogen = dark blue, oxygen = red and sulfur = green).



Aim 2: To determine the structure of ERα/β LBD/GW5638 associated with GW 7β-16 and/or other CoRNR box peptides and to identify the specific interactions between GW5368 and ER that facilitate corepressor docking.

To fulfill this specific aim, we first screened out a condition that could crystallize the complex of GW-ERα LBD bound to GW 7β-16 and form shower crystals. However, after exhaustive trials of crystallization optimization, we were still unable to get diffraction quality crystals. The attempts included macro- and micro- seeding, soaking native crystals with different GW-selective and CoRNR box peptides etc. Alternatively, with a refined structure of GW-hERα-LBD, we were able to map the potential binding site of the peptide by computer modeling and used mutagenesis and the mammalian two-hybrid assay to test different models. We proposed two different models depicted in Figure 4.

The GW-selective 7β-16 peptide was modeled into a five-turn α helix with an amphiphilic feature. If H12 is rigid and stationary, the distinct orientation of H12 in the GW-ER is able to form a three-helix bundle with 7β-16 and H3 as shown in Model 1, with the interactions between the core motif (LXXLL) of the peptides and the exposed Leu-rich region in H12. One could expect that changing these residues to charged amino acids would disrupt the interaction. However, we did not see a reduced binding affinity when we mutated Leu536, Leu539 and/or Leu540 to Arg. Instead, a five-fold enhanced interaction was observed in the triple Leu mutant by the mammalian two-hybrid assay (Figure 5A), suggesting that the first model (Figure 4A) might not be correct.

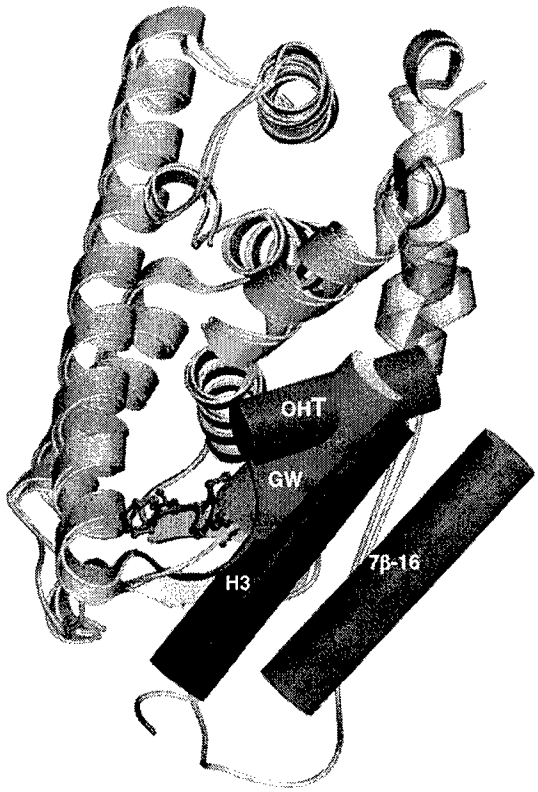
However, we found that deletion of H12 (ERα 537X or ERα 538X), which was previously shown to increase NCoR binding to ERα (Webb et al., 2003), enhanced the binding of GW-selective peptides to GW-ERα LBD (Figure 5B). This data suggests that GW-selective peptide might mimic the CoRNR box peptide to compete with H12 for the AF-2 cleft in GW-ERα. We are currently in the process of making other ER mutants such as L379R, K362A, L372R and V376R in the AF-2 cleft to test this hypothesis.

The technical details of performing mammalian two-hybrid assay are given as follows:

COS-7 cells were cultured in DMEM (Mediatech, Inc., Herndon, VA) supplemented with 10% fetal bovine serum (Atlanta biologicals, Lawrenceville, GA) and maintained in the 37°C incubator with 5% CO_2. The cells were plated into 48-well plates 24 hr before transfection. DNA was delivered to the cells by transient transfection using PolyFect. (Qiagen, inc., Valencia, CA). 100nM of ligands were added to the cells 18-24 hr before the cell harvest. For mammalian two-hybrid assay, 200 ng 5xGal4-TATA-Luc reporter plasmid, 200 ng VP16-receptor fusion, 200 ng Gal4-DBD-peptide fusion, and 20 ng normalization plasmid pRL-TK or pCMV-ßgal were used. 100nM of GW7604 were added to the cells 18-24 hr before the cell harvest. Luciferase activities were normalized to either Renilla luciferase of β-galactosiase activities by Dual-Luciferase Reporter Assay or β-galactosidase enzyme assay systems (Promega Corp., Madison, WI).

Figure 4. Upper panel shows the model that GW-selective peptide forms a three-helix bundle with H12 and H3. Lower panel shows GW 7β-16 peptide binding to GW-ERα LBD. H12 is omitted for clarity.

A



B

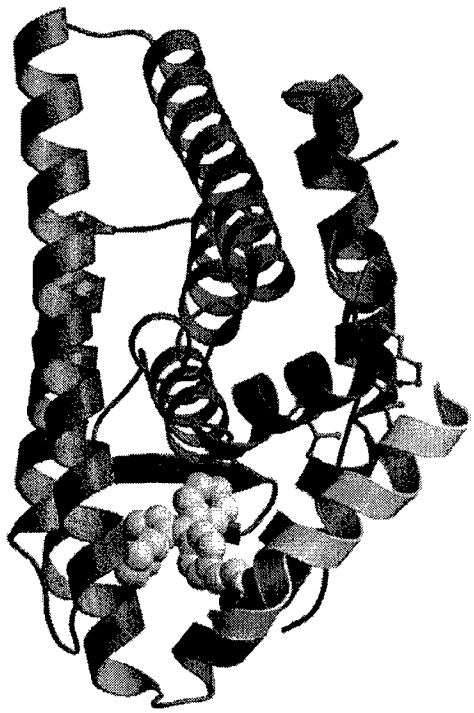
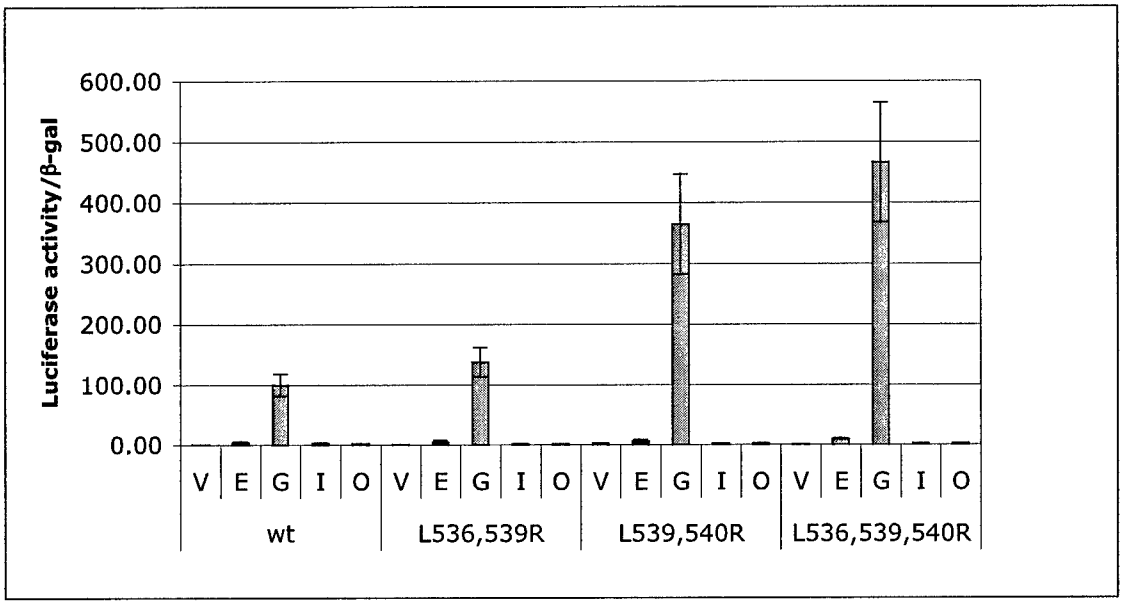
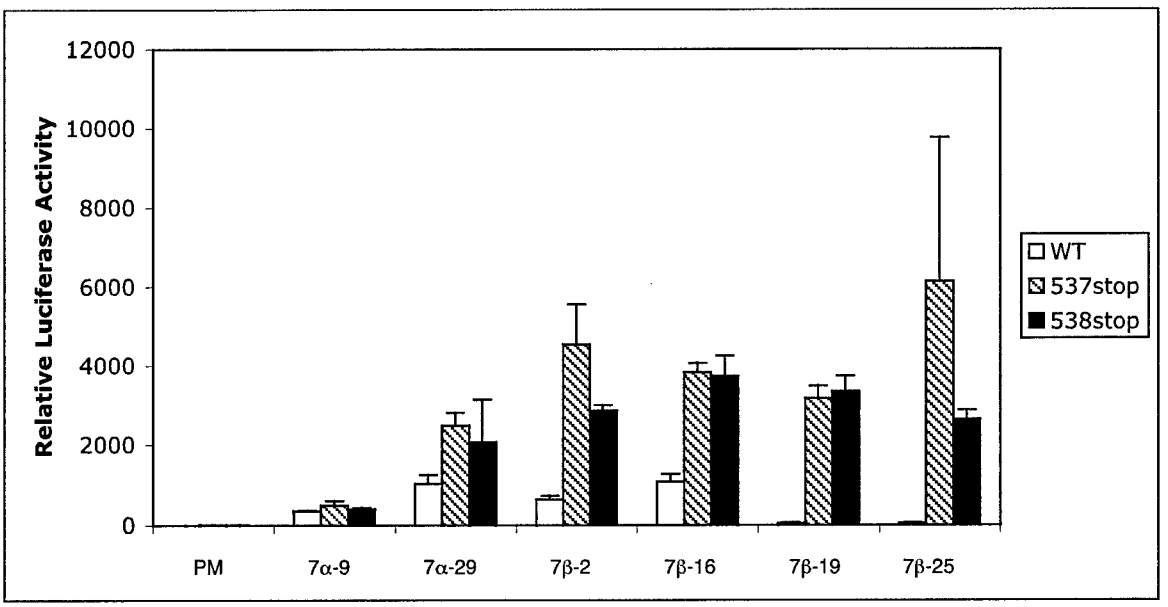


Figure 5. Mammalian-two hybrid data showing mutations in ERα affect the binding of GW-specific peptides

A



B



Aim 3: To determine the structure of C through E domains of human ERα/β and to characterize the interactions among these functional domains, which may have important pharmacological significance in biology and drug design.

I have attempted to express C through E domains of human ERα/β but it is problematic to purify the full-length of this region for further biochemical analysis and crystallization.

Due to the permission from my thesis committee to graduate by the end of the summer, I will no longer pursue aim 3 of this proposal.

KEY RESEARCH ACCOMPLISHMENTS

In the first year of the training, I have crystallized, determined and refined the atomic structure of ERα LBD in complex with GW5638 and revealed the mechanism that may account for the ability of GW5638 to inhibit tamoxifen-resistant breast tumor explants.

REPORTABLE OUTCOMES

1. The structure of GW-ERα LBD was deposited to the protein data bank (PDB ID: 1R5K) and a manuscript of this work is in submission.
2. The structural study of GW-ERα LBD was presented as a poster at the Nuclear Receptor Keystone Symposia (02/28/04 – 03/04/04) supported by Keystone Symposia, held at Keystone Resort, Keystone, Colorado.

CONCLUSIONS

The completion of aim 1 revealed that the crystal structure of GW-ERα LBD may explain why GW5638/7604 is a more potent antagonist and is an effective inhibitor of tamoxifen-resistant MCF-7 tumor explants. The data showed that relatively subtle ligand modifications could significantly alter the conformation of the H12 molecular switch. In addition to preventing coactivator recruitment by occlusion of the AF2 cleft, GW5638/7604 also destabilizes ERα. This effect is associated with a rotation of H12, which leads to an increase in the surface hydrophobicity of the ERα LBD. Problems encountered to obtain diffractable crystals with the GW-ERα-peptide complex and to generate C through E domains of ERα were also addressed. Alternative experiments done or proposed to substitute the original approaches under aims 2 were described in the report body section.

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RIVISED STATEMENT OF WORK

Aim 1: To determine the structure of human ERα/β-LBD in complex with GW5638 and to compare the conformational changes in ERα/β-LBD elicited by tamoxifen and GW5638.

1. To generate and purify bacterially expressed ERα/β-LBD/GW5638 complexes for biochemical analysis and crystallization (months 1-3).
2. To obtain single, diffraction-quality ERα/β-LBD/GW5638 crystals for x-ray analysis (months 4-12).
3. To determine the three-dimensional x-ray structure of ERα/β-LBD/GW5638 complex (months 13-18).

Aim 2: To determine the structure of ERα/β-LBD/GW5638 associated with GW 7β-16 and/or other CoRNR box peptides and to identify the specific interactions between GW5368 and ER that facilitate corepressor docking.

1. To generate and purify bacterially expressed ERα/β-LBD/GW5638 and prepare ERα/β-LBD/GW5638/peptide complexes for biochemical analysis and crystallization (months 12-16).
2. To generate ERα mutants based on the models of peptide-ERα interactions from GW-ER structure and establish the methodology of mammalian two-hybrid assay (months 17-20).
3. To detect the interaction of each corepressor-like peptide to ERα bound to tamoxifen, GW5638 and other ligands (months 18-24).